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Genetic combining ability of glucoraphanin level and other horticultural traits of broccoli†

Jason M. Abercrombie, Mark W. Farnham* & James W. Rushing
*U.S. Department of Agriculture, Agricultural Research Service, U.S. Vegetable Laboratory, and the Clemson University Coastal Research and Education Center, 2700 Savannah Highway, Charleston, SC 29414-5334, U.S.A.; (*e-mail: mfarnham@saa.ars-usda.gov)*

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Summary

Broccoli (*Brassica oleracea* L., Italica Group) is a source of glucosinolates and their respective isothiocyanate metabolites that are believed to have chemoprotective properties in humans. Glucoraphanin (4-methylsulfinyl-butyl glucosinolate) is a predominant glucosinolate of broccoli. Its cognate isothiocyanate, sulforaphane, has proven a potent inducer of phase II detoxification enzymes that protect cells against carcinogens and toxic electrophiles. Little is known about the genetic combining ability for glucosinolate levels or the types of genetic variation (i.e., additive vs. dominance) that influence those levels in broccoli. In this study, a diallel mating design was employed in two field experiments to estimate combining abilities for glucoraphanin content. The diallel population was developed by crossing nine doubled-haploid (inbred) parents in all possible combinations (36), excluding the reciprocals. Horticultural traits of all entries were assessed on a plot basis. In fall 2001, glucoraphanin concentration of broccoli heads ranged from 0.83 to 6.00 μmol/gdw, and in spring 2002, ranged from 0.26 to 7.82 μmol/gdw. In both years, significant general combining ability was observed for glucoraphanin concentration and total head content, days from transplant to harvest, head weight, and stem diameter. Conversely, no significant specific combining ability was observed for any trait in either year. Results indicate that a given inbred will combine with others to make hybrids with relatively predictable levels of head glucoraphanin as well as, other important horticultural traits. This should allow identification of inbreds that typically contribute high glucoraphanin levels when hybridized with others.

Abbreviations: GCA: general combining ability; SCA: specific combining ability

Introduction

Glucosinolates are sulfur-containing glycosides (β-thioglucoside *N*-hydroxysulfates, with an R-group that is an alkyl, alkenyl, thioalkyl, thioalkenyl, aryl, arylalkyl or indoyl moiety), which occur in cruciferous crops. In recent years, several epidemiological studies have suggested that isothiocyanates resulting from the hydrolysis of alkyl glucosinolates found in cruciferous vegetables may play a chemoprotective role in the human diet by reducing the risk of cancer (Hecht, 2000). Michaud et al. (1999) reported results from a large cohort study in which there was a significant correlation between cruciferous vegetable consumption and reduction in bladder cancer incidence. Other studies provide evidence that cruciferous vegetable consumption reduces the risk of cancers of the colon/rectum (Graham et al., 1978; Kohlmeir & Su, 1997; Verhoeven et al., 1997), prostate (Jain et al., 1999; Kolonel et al., 2000),

†Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

breast (Terry et al., 2001), and lung (London et al., 2000), as well as non-Hodgkins lymphoma (Zhang et al., 2000). Consequently, protective effects derived from consumption of *Brassica oleracea* L. vegetables such as broccoli (*Brassica oleracea*, Italica group) have attracted increasing attention.

Of particular importance to the protective effects of broccoli is sulforaphane, the cognate isothiocyanate of glucoraphanin (4-methylsulfinyl-butyl glucosinolate), a predominant glucosinolate in this crop. Sulforaphane is a potent inducer of mammalian detoxification and antioxidant (Phase 2) enzyme activity. It has been shown to protect against tumorigenesis in a rodent mammary tumor model (Fahey et al., 1997; Zhang et al., 1994; Zhang et al., 1992). There is considerable interest in understanding how to increase glucoraphanin levels in broccoli to enhance its chemoprotective capacity and add value for its development as a functional food (Farnham et al., 2004; Giamoustaris & Mithen, 1996; Kushad et al., 1999; Shapiro et al., 1998).

Faulkner et al. (1998) suggested that genetic factors responsible for high levels of the methylsulphinylalkyl glucosinolates in wild relatives of *B. oleracea* could be transferred to cultivated broccoli. They showed that hybrids formed by crossing inbreds and wild relatives express much higher Phase 2 enzyme induction potential than the broccoli inbreds themselves. Most recently, Mithen et al. (2003) reported enhanced isothiocyanate production in broccoli after introgression of three genomic segments of *Brassica villosa* L. into a more standard broccoli phenotype through several breeding cycles. This approach may ultimately yield a cultivar, however, broccoli lines resulting from *B. villosa* × *B. oleracea* populations require numerous generations of selection to bring the horticultural phenotype to commercial status. This drawback could be avoided by either altering the glucosinolate composition in broccoli through transformation of specific genes from the glucosinolate biosynthesis pathway into the crop (Li et al., 2001) or by exploiting the genetic diversity for glucoraphanin concentration found in relatively elite broccoli germplasm (Farnham et al., 2000) to breed new cultivars with high glucoraphanin.

Farnham et al. (2000) characterized glucoraphanin and Phase 2 enzyme induction potential of relatively elite and diverse broccoli inbred (doubled-haploid) lines that have horticultural traits of market quality. Results indicated a significant role of genotype in expression of glucoraphanin level in broccoli heads. The authors concluded that existing genetic variation could be used in a program of hybridization and rapid development of enhanced doubled-haploid lines. Kushad et al. (1999) examined a set of 24 F_1 hybrid and open-pollinated cultivars and 26 inbred lines of broccoli in a single environment and observed a range of glucoraphanin from 1.5 to 21.7 μmol/gdw in heads for cultivars and a range from 0.8 to 13.8 μmol/gdw for inbreds. Those authors also concluded that significant variation for glucoraphanin concentration existed in the elite germplasm they examined.

Diallel crossing systems have been used to study genetic variation of crop traits exhibiting quantitative inheritance and to provide a basis for a breeding strategy to improve such traits (Baker, 1978). Data derived from a diallel can be used to determine the mean performance, or the *general combining ability* (GCA) of a given line in hybrid combinations with all other lines. Individual crosses may vary significantly from that expected based on the mean performance of the lines (Falconer, 1981). In such cases, certain hybrid combinations exhibit *specific combining ability* (SCA) (Griffing, 1956). Combining ability estimates are useful for identifying superior parents for cultivar development (Fehr, 1987).

To our knowledge, only one previous study describes the use of a diallel cross population with broccoli. Hulbert and Orton (1984) evaluated genetic and environmental effects on mean maturity date and uniformity in broccoli for fourteen hybrids formed by crossing six commercial inbreds. They chose only inbreds that were morphologically similar to one another to serve as parents. Hulbert and Orton (1984) observed a preponderance of specific combining ability variances 30 times that of general combining ability for uniformity. However, general combining ability mean squares were over 20 times those of specific combining ability for mean maturity (Hulbert & Orton, 1984). The authors concluded that breeding for highly uniform hybrids would necessitate identification of specific crosses giving the best uniformity. Conversely, the general performance of a parent in different crosses would allow one to make a hybrid with desirable and predictable maturity.

The objectives of this study were: to utilize elite and diverse lines to develop a diallel population expressing variation for certain horticultural traits and glucoraphanin level; to use this diallel population to estimate general and specific combining ability for those traits; to identify crosses that produce high levels of glucoraphanin; and to compare the total content of glucoraphanin in a given head to the head glucoraphanin concentration. The goal of the last objective

was to assess the relative importance of glucoraphanin quantity (i.e. the full chemoprotective potential) versus glucoraphanin concentration.

Materials and methods

Plant materials

Forty-five entries were evaluated in this study, including nine inbred (doubled-haploid) lines developed at the U.S. Vegetable Laboratory and a diallel population of 36 F_1 hybrids made using the inbreds. The nine parental inbreds represent a diverse phenotypic and genotypic sample of broccoli derived from relatively elite sources (Farnham et al., 2000). These nine parents were crossed in all possible combinations, ignoring reciprocals, to form the diallel population of 36 hybrids.

Plant culture

In 2001, all forty-five entries were seeded to a commercial potting mix (Metromix 200, Grace Sierra, Milpitas, CA) in trays in a greenhouse during the first week of August and transplanted to the field on 19 September. The design of the field trial was a randomized complete block with three replications. Individual plots consisted of a single row of eight to twelve plants of an entry. Spacing between rows was 102 cm, and spacing between plants within a row was 15 cm throughout the field. In 2002, all entries were seeded to potting mix the first week of February and transplanted to the field on 5 March. This spring field trial was conducted the same as in the fall. All cultural practices (e.g., cultivation, fertilization, and irrigation) for both trials were standard for local conditions (Farnham et al., 2000). The soil type at the Charleston site is a Yonges loamy sand (fine loamy mixed, thermic Albaqualfs).

Head harvest and horticultural trait evaluation

As plots approached maturity, trials were checked every 2–3 days to identify plants ready for harvest. In all trials and with all entries, heads were evaluated and harvested when head diameter reached 10–12 cm. Three heads per plot were sampled at random, and subtending stalks were cut to a 15-cm length. Sampled heads were weighed and stem diameter of the cut stem was measured. Sample dates were recorded for calculation of the mean number of days from transplant to harvest (DTH) for a given plot. Heads were immediately placed on ice and within 30 min of field harvest, florets were cut from the stem, placed in an individual sealable freezer bag, and frozen at −80 °C. During each harvest, horticultural traits (i.e., plant height and width, head position, firmness, color, bead size, etc.) were recorded for six random plants in each plot.

Glucosinolate extraction and sample preparation

Frozen florets were lyophilized, ground into a fine powder using coffee grinding mills, and stored at −80 °C. Glucosinolates were extracted from a 1.0 g sample of the freeze-dried tissue using 25 ml of boiling 70% MeOH for 10 min. Samples were intermittently agitated during extraction. Crude extracts were filtered using Whatman no. 2 filter paper, volume was adjusted to 25 ml, and extracts were stored at −20 °C until analyzed. Immediately prior to HPLC analysis, extracts (2 ml) were centrifuged for 10 min. Solid phase extraction of resulting supernatants was performed using 1.5 ml C18 Extract-Clean™ columns (Alltech Associates, Inc., Deerfield, IL). Cleaned samples (1.75 ml) were concentrated using a rotary evaporator, and volumes were readjusted back to 1.75 ml by adding HPLC grade acetonitrile. These samples were vortexed for 5 s and placed in a capped HPLC vial.

HPLC analysis

The glucosinolates were analyzed on a Shimadzu Class VP HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD) consisting of two pumps, an autosampler, and a diode array detector set at 235 nm. Separation was performed as described by Troyer et al. (2001). Glucoraphanin and glucoiberin were identified in unknowns using purified standards supplied by M. Berhow and S. Vaughn (USDA-ARS, Peoria, IL). The amount of glucoraphanin present in the samples was determined by a standard curve generated using four concentrations of sinigrin monohydrate (Sigma-Aldrich Co., St. Louis, MO) and expressed in μmol/gdw. On average, glucoiberin amounted to less than 5% of the glucoraphanin peak and was not detected in over half of the samples. Thus, we did not conduct analysis of variance on the glucoiberin quantity.

Statistical analysis

Analyses of variance (ANOVA) of the subset of replicated samples from fall 2001 and spring 2002 trials were performed using the PROC GLM of SAS (release

6.12.SAS Inst., Inc., Cary, NC). Entry means were compared using Fisher's protected LSD. General (GCA) and specific (SCA) combining ability sums of squares were computed for glucoraphanin concentration, total glucoraphanin/head, stem diameter, head weight, plant height, plant width, and days from transplant to harvest (DTH) using method 4 described by Griffing (1956). These data were analyzed according to a fixed model (Griffing, 1956) where our concern was with comparisons of combining abilities of the actual parents used in the experiment and with the identification of superior combinations. Individual general combining ability and specific combining ability effects for crosses were calculated if their respective trait mean squares were significant ($P < 0.05$) for each year or the average across both years.

Results and discussion

Trait means among the nine parents used to form the diallel varied significantly (Table 1). Total glucoraphanin/head ranged from 11.00 to 162.16 (μmol/gdw), and glucoraphanin concentration ranged from 0.37 to 4.77 (μmol/gdw). These two traits were the most variable, with more than 10-fold differences between highest and lowest parents. Days from transplant to harvest ranged from 65 to 95 days, head weight ranged from 153 to 224 g, stem diameter ranged from 25 to 38 mm, and width from 67 to 92 cm. Of all traits, height varied the least among parents ranging from 48 to 56 cm. Most means were not significantly different for this trait.

A comparison of mean glucoraphanin concentration among parents used to make the diallel, respective half-sib families, and the mean of all other eight parents over both years (Figure 1) demonstrated that half-sib family means of relatively "low" parents (i.e., USVL089, USVL039, and USVL047) were typically higher than the "low" parent mean, yet lower than the mean of all other eight parents. The intermediate parents USVL012 and USVL032 were different in that their half-sib family means were higher than the mean of the other eight parents. This result might suggest the presence of high-parent heterosis or overdominance for crosses involving these parents. The three highest parents (USVL105, USVL066 and USVL048) displayed higher mean glucoraphanin concentrations than their respective half-sib families.

All traits were significantly affected by cross (Table 2), but significant environmental effects were observed only for days from transplant to harvest, head weight, and width (Table 2). Significant cross-by-environment interactions were observed for total glucoraphanin/head, glucoraphanin concentration, and stem diameter, but not for the other traits.

Results were variable in previous studies that evaluated the relative contribution of genetic versus environmental effects on glucoraphanin concentration in broccoli. Brown et al. (2002) found significant effects of genotype and genotype-by-environment interaction on glucoraphanin concentration for 10 broccoli genotypes grown over four environments; however, most variation was attributed to genotype, with environmental effects being non-significant. Farnham et al. (2004) observed significant genotype, genotype by environment, and

Table 1. Means of the nine diallel parents across two environments (Fall, 2001 and Spring, 2002) for the traits glucoraphanin concentration (GR), total glucoraphanin (GR/head), days from transplant to harvest (DTH), head fresh weight (HFWT), stem diameter (SD), height (HT), and width (WD)

Parental line	GR (μmol/gdw)	GR/head (μmol/head)	DTH (days)	HFWT (g)	SD (mm)	HT (cm)	WD (cm)
USVL105	4.77	162.16	76	224	38	55	79
USVL012	2.25	71.01	65	204	38	55	85
USVL089	0.96	27.78	69	199	31	51	74
USVL070	1.80	44.10	66	153	25	55	71
USVL039	0.67	16.41	80	165	29	56	83
USVL047	0.37	11.00	73	198	34	51	67
USVL066	4.20	105.89	95	174	34	48	73
USVL032	2.45	73.03	76	201	37	54	92
USVL048	3.29	98.26	81	217	33	53	78
$LSD_{0.05}$	1.25	40.77	4	29	3	6	6

Table 2. Mean squares from the analysis of variance of 36 F_1 crosses from the diallel among nine parents over two environments (Fall, 2001 and Spring, 2002) for the traits glucoraphanin concentration (GR), total glucoraphanin (GR/head), days from transplant to harvest (DTH), head fresh weight (HFWT), stem diameter (SD), height (HT), and width (WD)

		Mean squares						
Source	df	GR/head	GR	DTH	HFWT	SD	HT	WD
Environment (env.)	1	20,822.5	0.71	10,486.2**	50,753.3**	146.7	277.9	1,305.3*
Rep (env.)	4	6,067.5**	7.7**	13.4	1,377.2	28.5*	92.3*	149.3*
Crosses	35	16,699.0**	13.7**	17.0**	3,747.7**	57.1**	48.1*	130.9**
GCA	8	9,805.5**	8.1**	64.2**	1,879.6*	29.9*	17.7	50.4
SCA	27	703.3	0.55	2.11	257.5	3.6	5.5	14.1
Cross × env.	35	2,251.9*	2.0**	0.8	903.4	17.1*	27.4	42.0
Error	140	1,304.0	1.1	7.2	749.8	9.8	29.2	32.0
Total	215							

*,** Significant at the $P = 0.05$ and $P = 0.01$ probability levels, respectively.

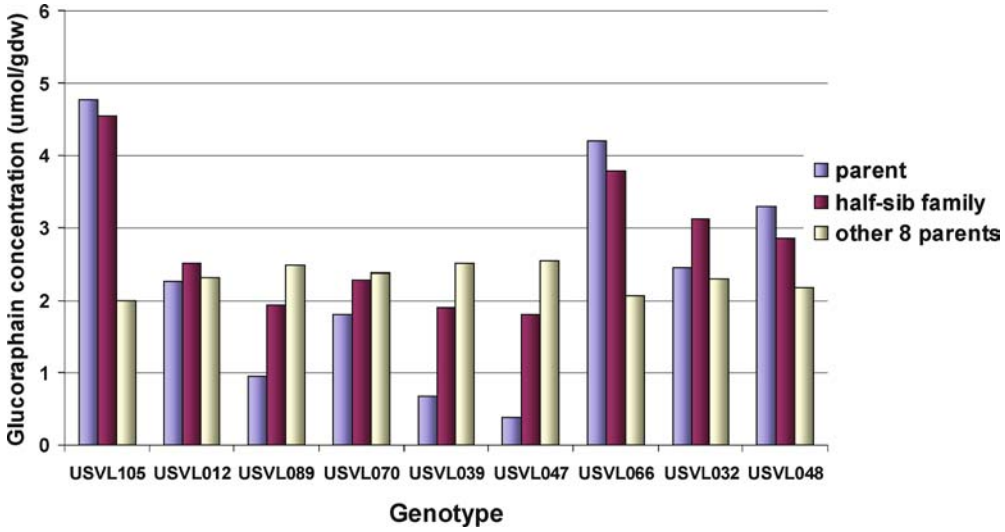

Figure 1. Mean Glucoraphanin concentrations for parents, their respective half-sib families, and all other eight parents crossed to each specific parent over two years (Fall, 2001 and Spring, 2002)

also environmental effects for glucoraphanin concentration of nine broccoli lines grown in three environments. Most recently, Vallejo et al. (2003) found that individual aliphatic glucosinolate levels were significantly affected by genotype, environment, fertilization, and all interactions of these factors.

Vallejo et al. (2003) reported glucoraphanin concentrations in the commercial cultivar, Marathon, in the late season that were 3-fold lower than values reported by Rosa and Rodrigues (2001) and 6-fold lower than glucoraphanin concentrations reported by Hansen et al. (1995). Similar differences have been observed between results from the current study and others we obtained previously. Specifically, six of nine diallel parents (USVL070, USVL039, USVL047, USVL066, USVL032, and USVL048) were previously evaluated in 1997 and 1998 (Farnham et al., 2004), and mean glucoraphanin values measured in the diallel tests are all lower than mean glucoraphanin concentrations from the 1997 and 1998 trials. These differences are probably due to environmental effects and genotype-by-environment interactions known to occur from research described above. In the present study, cross-by-environment interactions were significant, but environmental effects were not. This is likely due to the high number of hybrid crosses and the resulting abundance of mid-range values. In contrast, the previous study used mostly doubled-haploid (inbred)

Table 3. Estimates of general combining ability (GCA) effects for glucoraphanin concentration (GR), total glucoraphanin (GR/head), head fresh weight (HFWT), days from transplant to harvest (DTH), and stem diameter (SD), based on 36 crosses and 9 half-sib families derived from nine parents averaged across two environments

Parents	GR (μmol/gdw)	GR/head (μmol/head)	DTH (days)	HFWT (g)	SD (mm)
USVL105	2.0	72.1	1.3	13.6	1.3
USVL012	−0.3	−6.9	−5.0	8.3	2.5
USVL089	−1.0	−33.6	−1.8	−9.2	−2.6
USVL070	−0.6	−24.8	−2.7	−16.7	−2.5
USVL039	−1.0	−43.5	3.4	−27.7	−2.6
USVL047	−1.1	−28.0	−1.6	22.5	2.1
USVL066	1.1	30.8	4.1	−7.6	0.5
USVL032	0.4	15.2	0.8	3.7	0.9
USVL048	0.4	18.8	1.6	13.5	0.3

lines and relatively few hybrid crosses, resulting in a more variable population.

The combined two-year analysis of variance indicated that mean squares for GCA were significant for total glucoraphanin/head, glucoraphanin concentration, days from transplant to harvest, head weight, and stem diameter (Table 2). SCA was not significant for any trait. A separate analysis for each environment indicated very similar GCA effects (in terms of magnitude and sign; ±) for half-sib families for all parent/trait combinations except one (USVL 066 and stem diameter). In light of similar results between environments, a combined analysis was deemed appropriate (Table 3). A sizeable range was observed for the GCA effect of total glucoraphanin/head; –43.5 to 72.1, where USVL105 expressed the highest GCA and USVL039 displayed the lowest. USVL105 also exhibited the highest GCA effect for glucoraphanin concentration and head weight; 2.0 and 13.6, respectively. As expected, each genotype that exhibited positive GCA for glucoraphanin concentration also had positive GCA for total glucoraphanin/head. Conversely, each genotype with negative GCA for glucoraphanin concentration also displayed negative GCA for total glucoraphanin/head. General combining ability effects for days from transplant to harvest ranged from –5.0 to 3.4 and head weight ranged from –27.7 to 22.5.

These results show that GCA is more important than SCA in predicting crosses that produce high or low levels of glucoraphanin as well as, high or low amounts of total glucoraphanin/head. GCA mean squares for both total glucoraphanin/head and glucoraphanin concentration were approximately 14-fold greater than SCA mean squares. In addition, GCA mean squares were 30-fold greater than those for SCA with days from transplant to harvest. Hulbert and Orton (1984) reported a similar result from their diallel, with GCA mean squares 20-fold greater than those for SCA with maturity. This may indicate a predominant additive component to variation for maturity among crosses used in these studies. A 8-fold difference of GCA over SCA was observed for head weight and stem diameter. The fact that GCA mean squares were significant for all traits evaluated except height and width and that SCA mean squares were not significant for any trait indicates that many horticultural traits of a single cross progeny can be sufficiently predicted on the basis of GCA (Baker, 1978).

Significant GCA is hypothesized to result mainly due to additive gene effects, although non-additive effects (i.e. epistasis, dominance) may also occur (Baker, 1978). For the purposes of this study, epistatic effects are assumed non-significant. Thus, we conclude that the positive GCA effects for glucoraphanin concentration and total glucoraphanin/head in USVL105, USVL066, USVL032, and USVL048 indicate that selection of parents based upon their performance *per se* should effectively improve glucoraphanin concentration and total glucoraphanin/head in future hybrid combinations.

Nearly all broccoli consumed in the United States is harvested from hybrid production fields, therefore, it is essential to evaluate the expression of potentially beneficial traits in hybrid combinations (Farnham et al., 2004). This study has demonstrated the importance of GCA of broccoli parents in maximizing glucoraphanin concentration and optimizing other horticultural traits of broccoli in combination with glucoraphanin when

developing hybrids. Understanding this importance, broccoli breeders can more effectively enhance levels of glucoraphanin, improving the value of broccoli as a functional food, and possibly increasing its utility in nutritional and medical research.

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